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## Heterologous Expression and Secretion of a *Streptomyces scabies* Esterase in *Streptomyces lividans* and *Escherichia coli*

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The esterase gene from *Streptomyces scabies* FL1 was cloned and expressed in *Streptomyces lividans* on plasmids pIJ486 and pIJ702. In *S. lividans*, the esterase gene was expressed during later stages of growth and was regulated by zinc, as is seen with *S. scabies*. The 36-kDa secreted form of the esterase was purified from *S. lividans*. N-terminal amino acid sequencing indicated that the processing site utilized in *S. lividans* for the removal of the signal sequence was the same as that recognized for processing in *S. scabies*. Western blots (immunoblots) revealed the presence of a 40-kDa precursor form of the esterase in cytoplasmic extracts. A 23-amino-acid deletion was introduced into the putative signal sequence for the esterase. When this deleted form of the esterase was expressed in *S. lividans*, a cytoplasmic 38-kDa precursor protein was produced but no secreted esterase was detected, suggesting the importance of the deleted sequence for efficient processing and secretion. The esterase gene was also cloned into the pUC119 plasmid in *Escherichia coli*. By using the *lac* promoter sequence, the esterase gene was expressed, and the majority of the esterase was localized to the periplasmic space.

*Streptomyces scabies* is the causative organism for scab disease on a variety of underground vegetables (10). Infection by this gram-positive soil bacterium results in a proliferation of suberin at the site of lesion formation. Suberin is a waxy polyester compound normally produced by plants as a barrier to moisture loss and pathogen infection (23, 24). Suberin is composed of long-chain fatty acids, fatty alcohols, dicarboxylic acids, and phenolic compounds that are bound by ester and ether linkages (23, 24). Pathogen-produced enzymes that degrade suberin would most likely be secreted in order to interact with this insoluble matrix on the plant surface. An extracellular esterase which may be involved in suberin breakdown has been isolated and characterized from the pathogenic *S. scabies* strain FL1 (31).

In the streptomycetes and other gram-positive organisms, proteins that are targeted for secretion are released directly into the culture medium. In contrast, proteins that are secreted from gram-negative bacteria such as *Escherichia coli* are localized to the periplasmic space. Proteins targeted for secretion are typically synthesized in a precursor form which is processed during secretion (45). Processing involves the removal of a signal peptide from the amino terminus. While the amino acid sequence of the signal peptide is not conserved between secreted proteins, similar properties of these sequences have been recognized (50, 52). In general, there is a hydrophilic stretch of amino acids at the amino terminus of the signal sequence followed by a hydrophobic core of amino acids that precedes the cleavage site.

Most of the streptomycete secreted proteins studied thus far have been reported to contain signal peptides which show properties similar to those of signal sequences in other organisms. Examples of these proteins include endo- $\beta$ -N-acetylglucosaminidase H from *Streptomyces plicatus* (39), agarase from *S. coelicolor* (5),  $\alpha$ -amylase from *S. hygro-*

*scopicus* (19), and  $\beta$ -galactosidase from *S. lividans* (13). Common characteristics of the streptomycete signal sequences have been noted (11). *Streptomyces* spp. have been used as hosts for the expression of foreign proteins. For example, secretion of interleukin-1 $\beta$  (28), interleukin-2 (3), proinsulin (48), tumor necrosis factor (9), human CD4 (7), and *E. coli* R-TEM  $\beta$ -lactamase (36) was observed when these proteins were fused to a streptomycete signal sequence, although in some cases multiple forms of the foreign protein were detected.

When genes that encode secreted proteins from a variety of *Streptomyces* species are cloned and expressed in *S. lividans*, the gene products are typically secreted (5, 8, 12, 15, 25, 29, 34). In some cases, the site for signal sequence processing in *S. lividans* was demonstrated to be identical to that for processing in the native host strain (8, 25). A few genes for secreted streptomycete proteins have also been expressed in *E. coli*, where the gene products are typically found in the periplasmic space (19, 38, 47). These expression and secretion studies have suggested that the protein secretory pathways in the streptomycetes and in *E. coli* may be very similar, even though the important features of the streptomycete signal sequences have not been identified.

Secretion of the extracellular *S. scabies* esterase has been studied in more detail. A comparison of the amino acid sequence of the purified *S. scabies* esterase to the nucleotide sequence of the esterase gene (37) has suggested the presence of a 39-amino-acid signal sequence that is removed during secretion of this protein. To compare the process of secretion in *S. scabies* with that in other organisms, the esterase gene has been cloned and expressed in both *S. lividans* and *E. coli*. We have also begun to analyze some of the important features of the esterase signal peptide that are required for efficient secretion.

### MATERIALS AND METHODS

**Bacterial strains and plasmids.** Pathogenic *S. scabies* strain FL1 was isolated from a scab lesion on potato tuber as

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described previously (31). Strains C600 r-m+ (2) and JM109 (55) were used as the *E. coli* hosts for some of the cloning procedures and for expression of the esterase gene. *S. lividans* 1326 (4, 18) originated in the laboratory of David Hopwood, John Innes Institute, Norwich, England. Plasmids pIJ486 (54) and pIJ702 (21) are derivatives of the high-copy-number plasmid pIJ101 (22) and carry the gene for resistance to the antibiotic thiostrepton (*tsr*). pIJ486 also has a polylinker site useful for cloning DNA fragments of interest. pUC118 and pUC119 are pBR322 derivatives that carry resistance to ampicillin and have the *lac* promoter/operator sequence, the *lac* $\alpha$  complementation region, and a polycloning site (49). pGEM3Z is also a pBR322 derivative plasmid that contains resistance to ampicillin, the *lac* $\alpha$  expression sequence, a polycloning site, and promoters for the SP6 and T7 RNA polymerases (Promega).

**Media and growth conditions.** *S. scabies* was grown on oatmeal agar (44), and *S. lividans* was grown on R2YE (17) solid medium at 30°C for spore preparations. For plasmid preparations, the streptomycetes were grown in YEME medium (17) or in tryptic soy broth (Difco). When esterase production was required, the streptomycetes were grown in liquid minimal medium without polyethylene glycol (17) in flasks with springs to improve aeration. To induce esterase production, 2  $\mu$ M zinc sulfate was added to the medium (32). Thiostrepton at 5 or 20  $\mu$ g/ml was added to the liquid or solid medium, respectively, when necessary. Before inoculation into minimal medium, *S. lividans* spores were pregerminated. One plate of spores was harvested with 5 ml of sterile water, filtered through a cotton plug, and transferred to a 125-ml flask containing 0.25 ml of 1.0 M Tris, pH 9.0. The spores were heat shocked at 55°C for 10 min. The flask was cooled under tap water before 4 ml of double-distilled H<sub>2</sub>O, 0.5 ml of 10% yeast extract, 0.5 ml of 10% Casamino Acids, and 10  $\mu$ l of 5 M calcium chloride were added. The spores were then incubated with shaking at 37°C for 3 to 5 h. The culture was harvested by centrifugation, and the mycelial pellet was resuspended by vortexing in 5 ml of minimal medium before use as the inoculum. *E. coli* C600 and JM109 were grown in LB liquid medium (33), in MOPS (morpholinepropanesulfonic acid) minimal medium (35), or on antibiotic medium 2 agar plates (Difco). When needed, 25  $\mu$ g of ampicillin per ml or 0.4% lactose was added to the medium.

**Preparation of protein samples.** Fifty milliliters of *S. lividans* culture was harvested by vacuum filtration over Whatman no. 1 filter paper and crushed ice. The cells were washed with 50 ml of ice-cold sonication buffer (50 mM Tris [pH 8], 1 mM phenylmethylsulfonyl fluoride, 5 mM  $\epsilon$ -aminocaproic acid), resuspended in 1.5 ml of sonication buffer, and placed in dry ice-ethanol. Semifrozen cell suspensions were sonicated four times for 15 s each. Cell debris was removed by centrifugation at 12,000  $\times$  g for 5 min. Samples were stored at -80°C prior to analysis. Proteins from the culture filtrate were concentrated by precipitation with 10% trichloroacetic acid. The precipitate was collected by centrifugation, resuspended in sonication buffer, and stored at -80°C.

Both periplasmic and cytoplasmic fractions were isolated from *E. coli* cultures. To isolate periplasmic proteins, the osmotic shock or spheroplast procedure was carried out as described previously (46). Cytoplasmic extracts were prepared by sonically resuspending cell pellet after osmotic shock or spheroplast formation. Cell debris was removed by centrifugation. The resulting periplasmic and cytoplasmic protein samples were assayed for esterase activity as described previously, using *p*-nitrophenylbutyrate as the sub-

strate (32). The assays for alkaline phosphatase activity (46) and isocitrate dehydrogenase activity (20) were also carried out as described previously.

Protein concentration was determined by the method of Bradford (6).

**Immunoblot analysis.** Protein samples were mixed with sample buffer (62 mM Tris [pH 6.8], 10% glycerol, 1% sodium dodecyl sulfate [SDS], 0.125 M  $\beta$ -mercaptoethanol, 0.0005% bromophenol blue [final concentrations]) prior to electrophoresis on an SDS-10% polyacrylamide gel (1). Electrophoresis was carried out at a constant current of 20 mA for 8 h at room temperature. Proteins were transferred from the gel to nitrocellulose by using a semidry blotting apparatus and the 20% methanol buffer system described by Kyhse-Anderson (26). Molecular weight markers (Sigma SDS-70L) transferred to nitrocellulose were visualized by staining with Ponceau S in 1% acetic acid (41).

Filters with bound protein were washed (three times for 5 min each) in 200 ml of Tris-buffered saline (TBS; 20 mM Tris [pH 7.5], 500 mM NaCl) with 0.05% Tween 20. The filters were blocked by immersion in TBS with 3% gelatin and 0.05% Tween 20 for 30 min. Filters were incubated overnight with esterase antibody (1:1,000 dilution) in TBS with 1% gelatin and 0.05% Tween 20. The serum was isolated from rabbits immunized with purified esterase. Excess antibody was removed by washing the filters three times for 10 min each in TBS with 0.05% Tween 20. Filters were incubated with the second antibody (goat anti-rabbit alkaline phosphatase conjugate, 1:3,000 dilution; Bio-Rad) in TBS with 1% gelatin and 0.05% Tween 20 for 3 h. Blots were washed three times for 10 min each in TBS to remove residual Tween 20 and excess antibody. Color development was carried out according to the manufacturer's specifications (Bio-Rad). The molecular weights of the polypeptides were determined by comparing their relative migrations with those of the molecular weight markers.

**Native polyacrylamide gels.** Native polyacrylamide gels were run by using the discontinuous buffer system of Laemmli (27), with the omission of SDS in the buffers. The samples were heated at 70°C for 30 min to inactivate heat-labile esterases. Loading dye was then added to the samples (62 mM Tris [pH 6.8], 10% glycerol, 0.0005% bromophenol blue [final concentrations]) before the gel was loaded (12.5% running gel, 3% stacking gel). Gels were run initially at a constant current of 15 mA through the stacking gel and then at 20 mA for an additional 8 h at room temperature. Gels were assayed *in situ* for esterase activity by the procedure described by Rosenberg et al. (40).  $\alpha$ -Naphthyl acetate was used as the substrate.

**DNA manipulations.** Plasmid extraction from the streptomycete strains was carried out by the alkaline lysis procedure as described previously (17). *E. coli* plasmid DNA was purified essentially as described previously (42). DNA restriction fragments were purified from agarose gels by electroelution, using an IBI model UEA electroelution apparatus as specified by the manufacturer. DNA sequencing was carried out by the dideoxy-chain termination method (43), using a Sequenase kit from United States Biochemical. Restriction endonucleases, Klenow, and DNA ligase were purchased from either Bethesda Research Laboratories or Boehringer Mannheim Biochemicals and used according to the manufacturers' directions.

**RNA analysis.** RNA was isolated by the hot phenol method essentially as described previously (17). Cells were quickly chilled and filtered. The pellet was resuspended in lysis buffer (20 mM sodium acetate [pH 4.8], 0.5% SDS, 1 mM

EDTA) and broken by vortexing with glass beads and an equal volume of 60°C phenol that had been equilibrated with sodium acetate buffer, pH 4.8. After extraction with hot phenol and ethanol precipitation, the nucleic acid sample was treated with 33 U of DNase per ml in 50 mM Tris (pH 7.8)–50 mM MgCl<sub>2</sub> at 37°C for 1 h. RNA concentration was determined by  $A_{260}$  measurements.

RNA samples were denatured in 50% formamide–7.4% formaldehyde at 65°C for 20 min. The samples were dotted onto Gene Screen Plus membrane (New England BioLabs) according to the manufacturer's directions, and the filter was baked at 80°C for 2 h. Prehybridization and hybridization were carried out in 1% SDS–1 M NaCl–10% dextran sulfate at 60°C. The probe was a purified *Rsa*I fragment from the esterase gene and was labeled by the random priming method (14). The filters were washed twice in 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) at room temperature for 5 min each, twice in 2× SSC–1% SDS at 60°C for 30 min each, and twice in 0.1× SSC at room temperature for 30 min each.

**Protein sequence analysis.** All sequence analysis was carried out through the Molecular Biology Computing Center (College of Biological Sciences, University of Minnesota, St. Paul).

## RESULTS

**Expression of the *S. scabies* esterase gene in *S. lividans*.** The *S. scabies* esterase gene was originally cloned into plasmid pUC119 as a 2.5-kb *Sma*I fragment containing the entire coding sequence of the esterase gene and about 1 kb of flanking sequence (37). The resulting constructs were designated pJW27 and pJW9. The orientation of the esterase gene in pJW27 is the same as that of the *lac* promoter and *lac*  $\alpha$ -complementation region (Fig. 1A). To express the esterase gene in *S. lividans*, an approximately 2.2-kb *Pst*I-*Sph*I fragment from pJW9 was ligated to *Pst*I- and *Sph*I-cut pIJ702 (21) to make pJLS207 (Fig. 1B). In addition, the esterase was cloned into the pIJ486 vector (54). This was accomplished in two steps. The *Sst*I-*Eco*RI fragment from pJW9 was ligated to pIJ486 cut with *Sst*I and *Eco*RI. The resulting construct was cleaved with *Eco*RI and ligated to a purified *Eco*RI fragment from pJW27 in order to reconstitute the intact esterase gene, designated pJLS250 (Fig. 1C).

The pJLS207 and pJLS250 constructs were introduced into *S. lividans* 1326 (4, 18). In both cases, the esterase gene was expressed and the esterase protein was secreted into the growth medium. Estimates of esterase production have indicated that about 100 mg of protein per liter of culture can be produced in *S. lividans*. The amount of secreted esterase made depended on the growth conditions and the growth stage of the culture. Culture growth and esterase production were monitored for 24 h after the medium was inoculated with pregerminated spores (Fig. 2A). Esterase production significantly increased as the culture approached late-log- or early-stationary-phase growth. The addition of 2  $\mu$ M zinc sulfate to the medium enhanced esterase production six- to eightfold when compared with production in the culture grown without zinc. This effect of zinc on esterase production is similar to zinc induction of esterase expression observed for the esterase gene in *S. scabies* FL1 (Fig. 2C).

RNA dot blots were carried out to quantitate the amount of esterase mRNA produced in *S. lividans* harboring the pJLS250 plasmid in the presence and absence of zinc (Fig. 2B). The level of esterase transcript relative to total cellular RNA was about sixfold higher when cells were grown with

zinc than when they were grown without zinc. This increase in esterase mRNA paralleled the observed zinc-dependent increase in esterase protein during culture growth (Fig. 2A). The effect of zinc on esterase mRNA levels in *S. scabies* was also evaluated by RNA dot blots (Fig. 2D). The increase in esterase mRNA seen in zinc-grown cultures was similar to the observed increase in esterase enzyme activity (Fig. 2C). These results have suggested that expression of the *S. scabies* esterase gene is regulated by zinc in *S. lividans* and that the mechanism of zinc regulation involves changes in the levels of esterase mRNA. This is similar to the enhancement of esterase mRNA by zinc that is seen for esterase gene expression in *S. scabies*.

**Secretion of the esterase from *S. lividans*.** The esterase secreted from *S. lividans* carrying the pJLS207 construct was purified from the culture filtrate by using DEAE-Sephadex chromatography as described previously (32). The amino-terminal sequence of the purified esterase was determined by Edman degradation (53) and was found to be identical to the amino-terminal sequence of the esterase expressed and secreted from *S. scabies* FL1 (37). These results indicated that processing and secretion of the esterase from *S. lividans* was similar to esterase processing and secretion in *S. scabies*.

The N-terminal sequence of the esterase is shown in Fig. 3. The DNA sequence of the esterase gene and the amino-terminal amino acid sequence of the secreted protein predicts that a 39-amino-acid signal peptide is removed during secretion (37). If the esterase protein is posttranslationally processed, then an unprocessed form of the esterase which is about 4 kDa larger than the secreted esterase should be detectable in cytoplasmic extracts. Western blotting (immunoblotting) was used to detect esterase protein in both cytoplasmic and culture filtrate samples of *S. lividans* harboring plasmid pJLS250 (Fig. 4). In the culture filtrate, only one esterase species, with a molecular weight of about 36,000, was detected; this molecular weight is identical to that of the *S. scabies*-secreted enzyme. In the cytoplasmic sample, two forms of the esterase were detected. An esterase species with a molecular weight of about 40,000, corresponding to the predicted unprocessed size of the protein, was detected. The 36,000-molecular-weight species is probably the processed form of the esterase, since it comigrates with the esterase species in the culture filtrate. Detection of the processed form of the esterase in the cell extract could be due to residual processed esterase isolated with the cells or to rapid processing of the precursor esterase during the extraction procedure. Detection of the 40,000-molecular-weight form of the esterase indicated that the esterase was made as a precursor protein, with translation initiation at one of the two methionines indicated in Fig. 3, and that secretion of the esterase involved removal of a 39-amino-acid signal peptide. The data also suggest that secretion of the esterase occurs posttranslationally.

**Deletion within the signal sequence affects esterase secretion.** A hydrophilicity analysis (16) of the putative 39-amino-acid esterase signal peptide is shown in Fig. 3. Three distinct features of the signal peptide which are similar to those of other known signal peptides are seen (50, 52). The n region (N-terminal region) is hydrophilic in character, is 18 amino acids in length, and has four positively charged amino acids. The h region (hydrophobic region) contains the hydrophobic residues and is 15 amino acids in length. The c region (C-terminal region) lies between the hydrophobic domain and the cleavage site and is 6 amino acids in length. The proposed cleavage site itself follows the –1, –3 rule (51) for

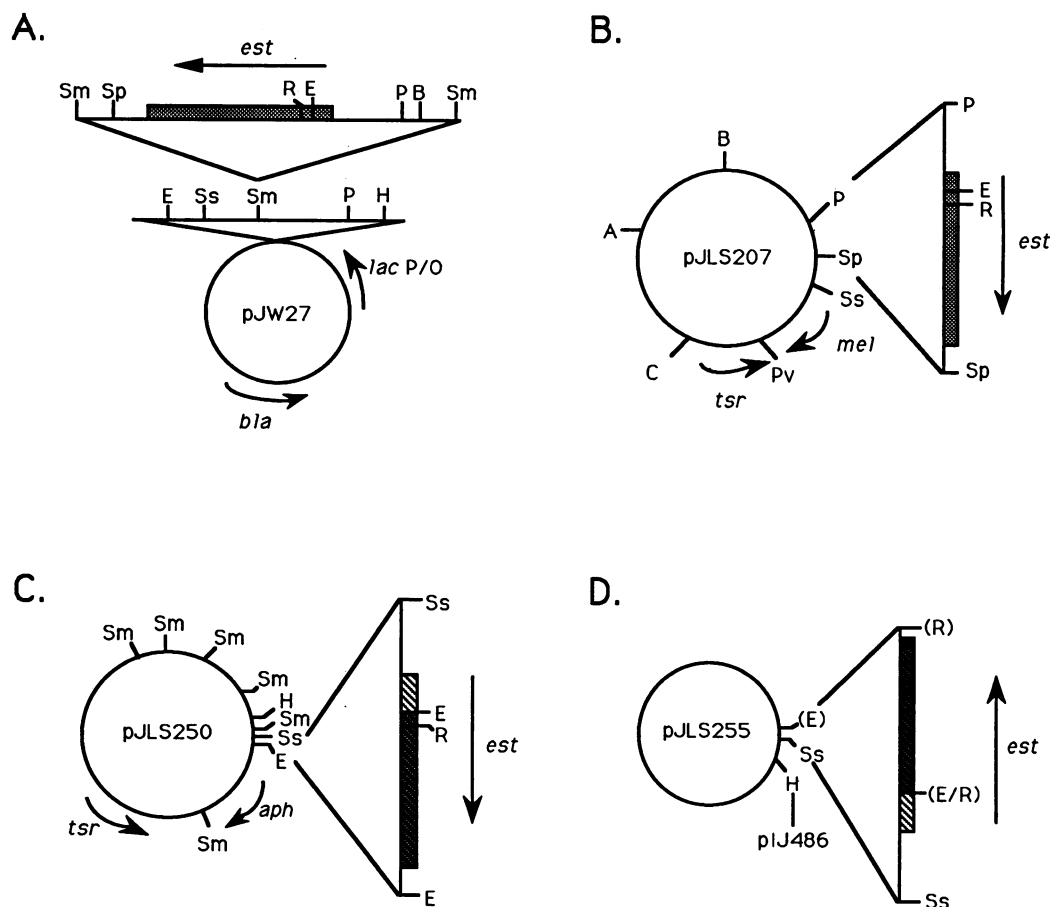


FIG. 1. Esterase gene constructs. (A) A 2.5-kb *Sma*I fragment containing the esterase gene (*est*) was cloned into the *Sma*I site within the polycloning sequence of pUC119. The orientation of the esterase gene in pJW27 is the same as that of the *lac* promoter. The arrows indicate the direction of transcription, and the stippled box marks the location of the esterase-coding sequence. The ampicillin resistance gene is labeled *bla*. B, *Bam*HI; E, *Eco*RI; H, *Hind*III; P, *Pst*I; R, *Rsr*II; Sm, *Sma*I; Sp, *Sph*I; Ss, *Sst*I. (B) The *Pst*I-*Sph*I fragment from pJW27 was cloned into the *Pst*I and *Sph*I sites of pIJ702. The approximate locations of the thiostrepton resistance gene (*tsr*) and the tyrosinase gene (*mel*) are indicated. Selected restriction sites are as follows: A, *Apa*I; C, *Cla*I; Pv, *Pvu*II; the other sites are defined in the panel A legend. (C) A 1.1-kb *Sst*I-*Eco*RI fragment from pJW9 (orientation of the esterase gene in pUC119 is opposite to that in pJW27) containing the 5' region of the esterase gene was ligated to the *Sst*I and *Eco*RI sites of pIJ486 (white striped box). A 1.5-kb *Eco*RI fragment from pJW27 containing the 3' half of the esterase gene was then cloned into the *Eco*RI site (dark striped box). The construct which reconstituted the intact esterase gene was designated pJLS250. The locations of the *tsr* gene and the aminoglycoside phosphotransferase (*aph*) gene are indicated. Selected restriction sites are shown and are defined in the panel A legend. (D) An *Sst*I-*Eco*RI fragment containing the 5' region of the esterase gene (white striped box) was cloned into the *Sst*I and *Eco*RI sites of pGEM3Z. This plasmid was cut with *Eco*RI, and the ends were filled in and ligated to a filled-in *Rsr*II fragment from the esterase gene (dark striped box). Plasmid pIJ486 was ligated to the *Hind*III site of this plasmid to make the bifunctional replicon pJLS255.

a signal peptidase I type of signal sequence in having alanines at both of these positions. The importance of these various regions for protein secretion in the streptomycetes has not been established.

To determine whether the signal peptide is required for efficient secretion, a 63-bp deletion was introduced into the esterase gene between the internal *Eco*RI and *Rsr*II sites. This region corresponds to a portion of the n region and all of the h region of the signal peptide (Fig. 3). The deletion construct removed 23 amino acids from the signal peptide and inserted a cysteine residue in its place. The region of DNA around the deletion site was sequenced to confirm that no other changes to the sequence were introduced and that the reading frame of the esterase gene was maintained (data not shown). The resulting construct, pJLS255, is a bifunc-

tional replicon capable of replication in both *E. coli* and the streptomycetes (Fig. 1).

Plasmid pJLS255 was introduced into *S. lividans* 1326. With this construct, no detectable esterase activity was found either in the culture filtrate or in cell extracts. Western blotting was used to detect potentially inactive forms of the esterase (Fig. 4). In the culture filtrate, no evidence for the presence of an inactive form of the esterase was apparent. However, in the cell extract, a polypeptide that had a molecular weight of about 38,000 was found. This is the predicted size of the unprocessed protein made from the deletion construct. The amount of this putative 38-kDa precursor esterase detected in cells containing plasmid pJLS255 was similar to the amount of 40-kDa precursor esterase detected with the pJLS250 construct (Fig. 4). There

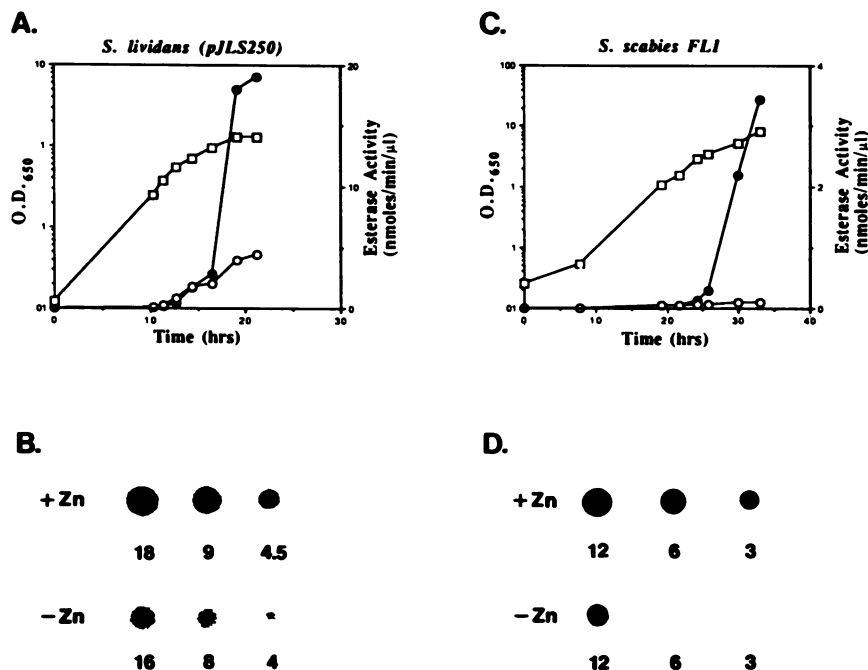


FIG. 2. The effect of zinc on esterase production during growth. Growth of *S. lividans* carrying plasmid pJLS250 (A) or *S. scabies* FL1 (C) in minimal medium with or without added zinc was monitored by optical density at 650 nm (O.D.<sub>650</sub>) (□). Only one growth curve for each organism is shown, since growth of the plus and minus zinc cultures was nearly identical. Esterase activity in cultures grown with (●) or without (○) zinc is expressed as nanomoles per minute per microliter of culture filtrate. The RNA dot blots for the *S. lividans*(pJLS250) and *S. scabies* FL1 cultures are shown in panels B and D, respectively. RNA was extracted from the *S. lividans* culture at an OD<sub>650</sub> of 1.3 and from the *S. scabies* culture at an OD<sub>650</sub> of 6.0. The total amount of cellular RNA dotted from the sample is indicated in micrograms below the dots. The blot in panel B was quantitated by the AMBIS Radioanalytic Imaging System; the blot in panel D is an autoradiogram.

was no evidence for the accumulation of excess unprocessed esterase inside the pJLS255-containing cells. These results indicated that removal of the hydrophobic core of amino acids from the signal peptide prevented esterase secretion and that this sequence of amino acids is critical for efficient secretion of the esterase.

Expression of the esterase gene in cells containing plasmid pJLS255 was also studied by RNA dot blot hybridization to a labeled esterase gene probe. The amount of transcript was similar to the amount of esterase RNA detected with *S. scabies* FL1, relative to total cellular RNA (data not shown). This result indicated that the inability to detect secreted esterase activity with cells carrying the pJLS255 construct was not due to lack of esterase mRNA synthesis but rather was due to the lack of esterase protein processing and secretion.

#### Expression and secretion of the *S. scabies* esterase in *E. coli*.

In the pJW27 construct, the esterase gene is positioned in the same orientation as the *lac* promoter/operator region (Fig. 1A). Cells harboring this plasmid produced an esterase activity coded by the *S. scabies* gene. A periplasmic fraction and a cytoplasmic fraction were prepared from the pJW27-containing cells. These fractions were assayed for esterase, isocitrate dehydrogenase, and alkaline phosphatase activities (Table 1). While the majority of the alkaline phosphatase activity was found in the periplasm, the isocitrate dehydrogenase activity was predominantly localized to the cytoplasm, as expected. These results verified the efficiency of the procedures used to separate the periplasmic and cytoplasmic fractions. The majority of the esterase activity was found in the periplasmic sample. No esterase activity was detected in the culture filtrate (data not shown).

The esterase species in the periplasmic fraction were detected by analyzing the periplasmic proteins on a non-denaturing polyacrylamide gel with an *in situ* esterase activity assay (Fig. 5). Three esterase activities were detected in the *E. coli* periplasmic sample. These esterases are coded by the *S. scabies* esterase gene in plasmid pJW27, since cells lacking this cloned gene do not show production of these heat-stable esterases (data not shown). The fastest-migrating esterase activity from the *E. coli* sample appeared to comigrate with the secreted esterase from both *S. scabies* FL1 and *S. lividans* harboring plasmid pJLS250, suggesting that a similarly processed esterase may be produced in *E. coli* cells. How the other two esterase species in the *E. coli* periplasmic sample are generated is not currently known. They could represent unprocessed esterase, esterase processed at an alternate site, or products of protease cleavage of the esterase. It is also feasible that an alternate start site for protein synthesis may be utilized by the translational apparatus in *E. coli*, resulting in the production of a differently sized but still functional esterase. The results of these experiments have suggested that since the majority of the *S. scabies* esterase produced in *E. coli* is secreted to the periplasm, some features of the streptomycete signal peptide are recognized by the *E. coli* processing pathway which targets the esterase for secretion.

#### DISCUSSION

The *S. scabies* esterase gene was cloned and expressed in *S. lividans*. Several features of esterase gene expression in *S. lividans* were similar to those observed with *S. scabies*. The appearance of the esterase and esterase-specific mRNA

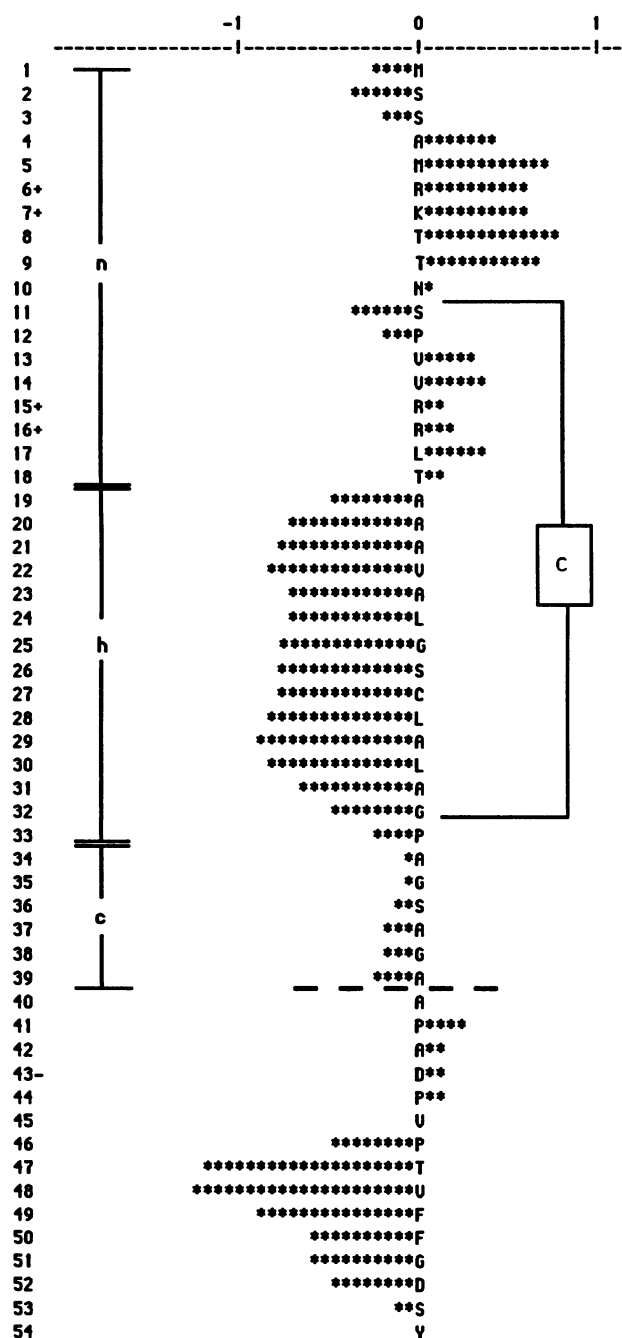


FIG. 3. Hydrophilicity analysis of the proposed esterase signal sequence. The algorithm of Hopp and Woods (16) was used for the analysis. The amino acids are numbered from the N terminus, starting with the first possible methionine. The charged residues are indicated with a plus or minus sign. The dashed line between residues 39 and 40 marks the proposed cleavage site for signal sequence removal. The amino acids deleted in the pJLS255 construct are within the bracket and are replaced by a Cys residue (C). The n, h, and c regions within the signal sequence are indicated. The number of asterisks next to each amino acid indicates the extent of its hydrophilic or hydrophobic character.

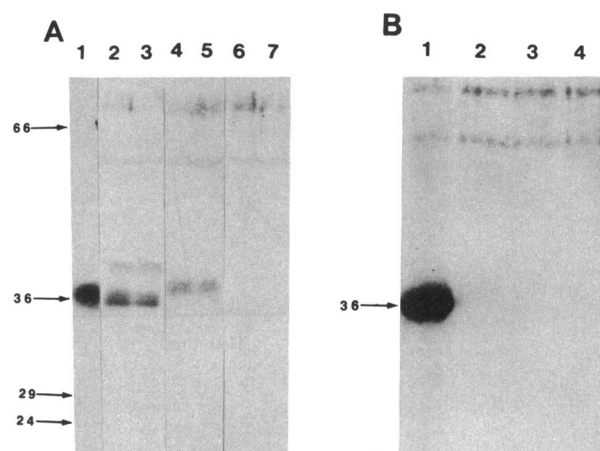


FIG. 4. Detection of cytoplasmic and secreted forms of the esterase. Proteins from cytoplasmic extracts (A) or from culture filtrates (B) were run on an SDS-10% polyacrylamide gel, transferred to nitrocellulose, and reacted with esterase-specific antibody. (A) Protein extract from cells harboring pJLS250 (lanes 2 and 3), pJLS255 (lanes 4 and 5), and pIJ486 (lanes 6 and 7); lane 1 contains secreted esterase purified from *S. scabies* FL1. (B) Culture filtrate from cells containing pJLS250 (lane 1), pJLS255 (lanes 2 and 3), and pIJ486 (lane 4). Molecular masses (in kilodaltons) of the markers are indicated.

occurred when cells were in the late-log or early-stationary phases of growth. This pattern of expression was the same when glycerol was used as the carbon source instead of glucose (data not shown), indicating that the timing of expression was not affected by glucose catabolite repression, as has been observed for the synthesis of other compounds in streptomycetes (30). This growth-stage-dependent expression pattern suggests that some factor required for esterase gene expression may not be available until later stages of growth or that a repressor of expression may be present during active mycelial growth.

Expression of the esterase gene in *S. lividans* was also stimulated by zinc, as originally observed for *S. scabies* (32, 37). Typically, a five- to eightfold increase in esterase production in *S. lividans* is observed when cells are grown in the presence of 2  $\mu$ M zinc sulfate. This induction is less than the 20- to 50-fold induction of esterase production by zinc observed for *S. scabies* (32). This difference may be due to the use of a nonoptimal concentration of zinc sulfate for esterase induction in *S. lividans*, or possibly a factor required for normal induction of the esterase is lacking in this *S. lividans* construct. Expression of the esterase gene is,

TABLE 1. Localization of alkaline phosphatase, isocitrate dehydrogenase, and esterase enzyme activities in *E. coli*<sup>a</sup>

Enzyme	Total activity (%) <sup>b</sup>	
	Cytoplasm	Periplasm
Alkaline phosphatase	4.6	95.4
Isocitrate dehydrogenase	82.2	17.8
<i>S. scabies</i> esterase	18.5	81.5

<sup>a</sup> *E. coli* C600 carrying plasmid pJW27 with the cloned esterase gene was used for these assays. The periplasmic fraction in this experiment was prepared by the spheroplast procedure.

<sup>b</sup> These results are from one experiment. Additional experiments using either the osmotic shock or spheroplast procedure gave similar results.



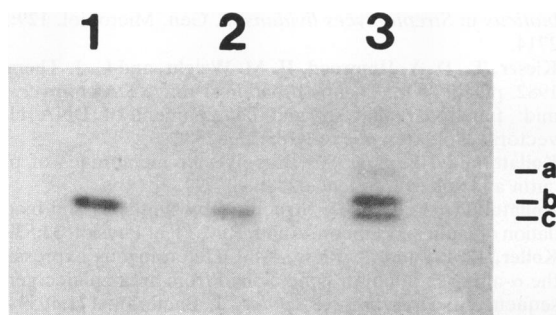


FIG. 5. Detection of active esterase species. Protein samples from either culture filtrates of the streptomycetes (lane 1, *S. scabies* FL1; lane 2, *S. lividans* with pJLS250) or the periplasm of *E. coli* (lane 3, C600 with pJW27) were run on a 12.5% polyacrylamide gel and assayed for esterase activity with  $\alpha$ -naphthylbutyrate and diazo blue. The three esterase activities detected in lane 3 are labeled a to c. The c band in lane 3 comigrates with the esterase activities in lanes 1 and 2.

however, stimulated by zinc, indicating that some of the factors required for zinc regulation must be contained within the cloned DNA fragment or are present in the *S. lividans* host strain.

The esterase gene from *S. scabies* was efficiently secreted into the culture medium when expressed in *S. lividans* and was efficiently secreted into the periplasmic space when expressed in *E. coli*. The esterase signal sequence was recognized by the secretion pathway in both of these organisms, suggesting potential similarities in the processing mechanism. In *S. lividans*, the processing site recognized for the esterase was the same as the cleavage site found in *S. scabies*. Other streptomycete proteins, such as the *S. tendae*  $\alpha$ -amylase inhibitor (25) and the *S. cacaoi* metalloprotease (8), have been expressed in *S. lividans* and processed at the same site as in the normal host strain. This similarity in processing site selection suggests potential similarities in the secretion pathways of *S. scabies*, *S. lividans*, and other streptomycetes.

When expressed in *E. coli*, the *S. scabies* esterase was secreted into the periplasm but migrated in a native polyacrylamide gel as multiple species, similar to the multiple processing sites suggested for secretion of the *S. plicatus* endo- $\beta$ -N-acetylglucosaminidase H in *E. coli* (47). Even though the streptomycete signal sequence is recognized by the *E. coli* processing pathway, there are obvious differences that result in cleavage at multiple sites and secretion of several protein species into the periplasmic space.

Except for their lengths, the streptomycete signal sequences are similar to other known signal peptidase I signal sequences (11, 52). Aside from sequence comparisons, little work has been done to identify the important features of the streptomycete signal sequences required for efficient secretion. An examination of the esterase signal peptide revealed features of the signal sequence common to other streptomycete secreted proteins and common to other signal peptidase I signal peptides (11, 52). A typical positively charged n region at the amino terminus of the signal sequence precedes a hydrophobic core of amino acids (h region). The esterase n region is 18 amino acids in length and has four positively charged residues. For the streptomycete proteins that have been characterized, the hydrophobic core is 12 to 28 amino acids in length (11). The esterase h region is 15 residues long. A c region is positioned between the hydro-

phobic core and the cleavage site. Even though the length of the c region may vary from 0 to 12 amino acids for the reported streptomycete secreted proteins, the sum of the h-region and c-region amino acids is fairly consistent and is usually about 24 amino acids. Small and uncharged amino acids, usually alanine or glycine, are typically found in the -1 and -3 positions relative to the cleavage site. The *S. scabies* esterase has 6 amino acids in the c region and has alanines in both the -1 and -3 positions, very typical for a signal peptidase I-like cleavage site.

The esterase signal sequence was analyzed for signal sequence cleavage sites by the method of von Heijne (51). Three cleavage sites were predicted on the basis of general properties of signal peptides and whether the cleavage site conformed to the -1, -3 rule. One of the predicted sites, between positions 39 and 40, is identical to the site that we have determined by comparing the nucleotide sequence of the esterase gene with the amino-terminal amino acid sequence of the secreted esterase. The second predicted cleavage site is between positions 42 and 43, 3 amino acids away from the observed cleavage site. A processed esterase species that would be consistent with cleavage at that site has not been detected. The third predicted site is between positions 31 and 32, near the end of the hydrophobic core of amino acids. Cleavage at this position may not be favored, since the sum of the lengths of the h region and c region in this case would be only 13 amino acids, significantly shorter than the typical 24-amino-acid length. Even though we have not detected an esterase species that corresponds to cleavage at that site, we cannot rule out the possibility that an initial cleavage occurs between positions 31 and 32, followed by a second cleavage between positions 39 and 40 to yield the only detectable form of the secreted esterase.

An interesting feature of the esterase signal sequence is a region of sequence that is identical to the signal sequence of the agarase from *S. coelicolor*. Ten amino acids within the 15-amino-acid hydrophobic region of the esterase signal peptide (AVALG- - -LAGPA) are identical to 10 amino acids within the 17-amino-acid hydrophobic sequence in the agarase signal peptide (5). These are the only two signal peptides for which we have found significant amino acid homology, even though the hydrophobic regions of signal sequences tend to be similar in having high frequencies of alanine, leucine, and valine residues. Although the significance of this sequence is not known at present, it is interesting to speculate that it may begin to define a preferred sequence of hydrophobic residues for efficiently secreted proteins. To better understand the mechanism of protein processing in the streptomycetes, current studies are focused on characterizing the effect of sequence changes within the signal peptide on the efficiency of secretion.

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